REMARKS

In response to the making of the restriction requirement FINAL, the non-elected claims have been cancelled.

The elected claims, claims 2-4, 7-8, 12-16 and 19-22, have also been cancelled and new claims 25-46 have been added to the application.

New claims 25-28 recite an isolated polynucleotide having a nucleotide sequence encoding 2-hydroxyisoflavanone synthase originating from leguminous plants which has an amino acid sequence having 55% or more, 70% or more, 80% or more and 90% or more, respectively, sequence identity to the sequence shown as SEQ ID NO:2 or an isolated polynucleotide having a nucleotide sequence complementary thereto.

New claim 29 recites an isolated polynucleotide having a nucleotide sequence encoding 2-hydroxyisoflavanone synthase originating from leguminous plants which has an amino acid sequence shown as SEQ ID NO:2 or has an amino acid sequence wherein one to 20 amino acids are substituted, deleted, added and/or inserted in the amino acid sequence shown as SEQ-ID-NO:2.

New claims 30-32 recite an isolated polynucleotide which has 70% or more, 80% or more, and 90% or more, respectively, sequence identity to the nucleotide sequence of 144-1712 of SEQ ID NO:1 and encodes 2-hydroxyisoflavanone synthase originating from leguminous plants and an isolated polynucleotide having a complementary sequence to the nucleotide sequences.

New claim 33 recites an isolated polynucleotide that has at least 15 contiguous nucleotides of SEQ ID NO:1.

New claim 34 recites a polynucleotide according to claim 33, which codes for the amino acid sequence of SEQ ID NO:2.

New claim 35 recites a recombinant DNA comprising a polynucleotide of any one of claims 25 to 34, which is connected to a regulation sequence that will express the polynucleotide in a sense direction.

New claim 36 recites a recombinant DNA comprising a polynucleotide of any one of claims 25 to 34, which is connected to a regulation sequence that will express the polynucleotide in an antisense direction.

New claims 37 and 38 recite a host cell transformed with the recombinant DNA according to claim 35 and claim 36, respectively.

New claim 39 recites a method for producing 2-hydroxyisoflavanone synthase comprising culturing a host cell that contains a polynucleotide encoding the amino acid sequence of SEQ ID NO:2.

New claim 40 recites a method for producing 2-hydroxyisoflavanone synthase comprising culturing the host cell according to claim 37.

New claims 41 and 42 recites a method according to claim 39 and 40, respectively, further comprising a step of collecting produced 2-hydroxyisoflavanone synthase.

New claim 43 recites a transgenic plant obtained by transforming the plant so that an amount of the product of the enzyme reaction catalyzed by 2-hydroxyisoflavanone synthase or derivatives thereof may be altered or increased by introducing the recombinant DNA according to claim 35 into a plant cell.

New claim 44 recites a transgenic plant obtained by transforming the plant so that an amount of the product of the enzyme reaction catalyzed by 2-hydroxyisoflavanone synthase or derivatives thereof may be altered by introducing the recombinant DNA according to claim 36 into a plant cell.

New claims 45 and 46 recite the transgenic plant according to claim 43 and 44, respectively, that is a leguminous plant.

The new claims are believed to be properly supported in the specification disclosure and to satisfy the requirements of 35 U.S.C. § 101 and 35 U.S.C. § 112.

Regarding claims 25-28, in the specification it is described that "[s]ubstantially comprising the amino acid sequence shown as SEQ-ID-No.:2" means that the amino acid sequence may include variations such as deletions, substitutions, additions and insertions in the amino acid sequence and that IFS of the present invention may contain such variations as long as the above-mentioned enzyme activity is maintained.

When a new amino acid sequence of cytochrome P450 is registered in a database, it is classified based on a sequence identity of the amino acid sequence to a known sequence. If the sequence has 40 % or more sequence identity to a known sequence, it is classified in the same family as the known sequence (namely the same CYP number, e.g., CYP93). Then, if the sequence has 55% or more sequence identity to a known sequence, it is classified in the same subfamily as the known sequence (namely the same CYP number +

alphabet, e.g., CYP93A,CYP93B, CYP93C). In most cases, cytochrome P450s belonging to the same subfamily have the same or substantially similar function. Although they may include an enzyme having a different function, it is a rare case.

Thus, a person of ordinary skill in the art would recognize that the description "the amino acid sequence which may include variation(s) such as deletion, substitution, addition and insertion in the amino acid sequence as long as the above-mentioned enzyme activity is maintained" shows that the inventors were in possession of "an amino acid sequence having 55 % or more sequence identity to the sequence shown as SEQ ID NO:2."

Proteins having a higher sequence identity have a higher probability of showing the same function. Accordingly, amino acid sequences with 70 or 80% or more sequence identity to CYP93C are those of IFS. Therefore, a person of ordinary skill in the art would also recognize that the present inventors were in possession of an "amino acid sequence with 70% or 80% or more sequence identity to the amino acid sequence shown in SEQ ID NO:2."

Regarding new claim 29 (supported by the description in the specification, inter alia, on page 3, lines 1-10) and claims 30-32

, it is apparent to those skilled in the art that there is a substantial likelihood that cDNA of cytochrome P450 having 70 % or more sequence identity encodes the enzyme having the same function at quite strong possibility. Furthermore, it would also be expected by the person of ordinary skill in the art that the amino acid sequence wherein 20 or less amino acids are substituted, deleted, added or inserted is that of an enzyme having the same function, since substitution, deletion, addition or insertion of 20 or less amino acids corresponds to 95% or more sequence identity. DNA sequences of CYP93C (IFS of soybean), CYP93C2 (IFS of licorice) and CYP93C17 (IFS of Lotus Japonicus) have about 80 % sequence identity.

New claims 33 and 34 find support in the specification, inter alia, on page 5, lines 6-15.

The remaining claims are believed to find proper support in the four corners of the specification.

As explained above, it is known to those skilled in the art that the new amino acid sequence is classified in the same or different subfamily as a known sequence depending on whether it has 55% or more sequence identity to the known sequence, and that

cytochrome P450 enzymes classified in the same subfamily have the same or quite similar function. Accordingly, there is a quite strong possibility that proteins belonging to CYP93C to which IFS of the present invention belongs and which have 55 % or more sequence identity to the sequence shown in SEQ ID No.2 are IFS. Actually, all amino acid sequences belonging to CYP93C and having function as reported are IFS.

Moreover, a sequence having more than 55 % sequence identity with the sequence of the polypeptide shown in SEQ ID No.2 is defined as that originating from leguminous plants, such sequence will always be concluded to be IFS. Although there is no evident proof therefor, generally the person of ordinary skill in the art can reach such a conclusion. If a homology search for an amino acid sequence encoded by DNA originating from a leguminous plant reveals more than 55 % sequence identity with the polypeptide of the present invention, the the person of ordinary skill in the art will without doubt deem the amino acid sequence to be the sequence of IFS. Of course, for obtaining his final conclusion, it will be necessary to confirm IFS function by expression of protein in yeast. However, this test can be performed by the method to

confirm the function of protein by expression p450 enzyme yeast, which method is well known to the skilled in the art and described for example in the following literature.

Fahrendorf T, Dixon RA., Stress responses in alfalfa (Medicago sativa L.). XVIII: Molecular cloning and expression of the elicitor-inducible cinnamic acid 4-hydroxylase cytochrome P450., Arch Biochem Biophys. 1993 Sep;305(2):509-15.

Urban P, Werck-Reichhart D, Teutsch HG, Durst F, Regnier S, Kazmaier M, Pompon D., Characterization of recombinant plant cinnamate 4-hydroxylase produced in yeast. Kinetic and spectral properties of the major plant P450 of the phenylpropanoid pathway., Eur J Biochem. 1994 Jun 15;222(3):843-50.

Akashi T, Aoki T, Ayabe S., CYP81E1, a cytochrome P450 cDNA of licorice (Glycyrrhiza echinata L.), encodes isoflavone 2'-hydroxylase., Biochem Biophys Res Commun. 1998 Oct 9;251(1):67-70.

Akashi T, Aoki T, Ayabe S., Identification of a cytochrome P450 cDNA encoding (2S)-flavanone 2-hydroxylase of

licorice (Glycyrrhiza echinata L.;Fabaceae) which represents licodione synthase and flavone synthase II., FEBS Lett. 1998 Jul 17;431(2):287-90.

In the meantime, it was also well known before the filing date of this application that a characteristic amino acid sequence of P450 is FxxGxxxCxG at a hem-binding region near the C-terminus. Hence, it is also well known that a polypeptide having the same sequence can function as p450 enzyme.

Thus, undue experimentation would not be necessary for confirming IFS function.

Referring to the prior art grounds of rejection, applicants arte submitting herewith an English language translation of applicants' priority application, Japanese patent application No. 11-63745, filed February 4, 1999. Both Steele et al. and the GenBank accession number AF135484 were published subsequent to this date. The filing of the English language translation of applicants' priority application perfects applicants' claim to priority and removes Steele et al. and GenBank accession number AF135484 as effective references against the claims of the present

application. The rejections based on these references, therefore, are no longer proper and should be removed.

As for Siminszky, Siminszky does not indicate that the sequence encodes IFS. Only Steele indicates that the sequence disclosed by Siminszky encodes IFS. Thus, the rejection based on Siminszky also fails and should be removed.

The foregoing is believed to be a complete and proper response to the Office Action dated March 13, 2003, and is believed to place this application in condition for allowance. If, however, minor issues remain that can be resolved by means of a telephone interview, the Examiner is respectfully requested to contact the undersigned attorney at the telephone number indicated below.

In the event that this paper is not considered to be timely filed, applicants hereby petition for an appropriate extension of time. The fee for any such extension may be charged to our Deposit Account No. 111833.

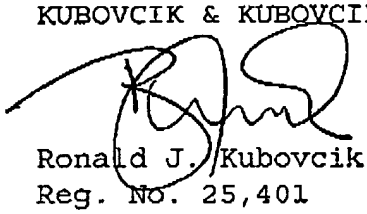
Checks in the amount of $465.00 for a three-month extension of the response period and $639.00 for excess claims (71) over those previously paid for (41) are attached.

PATENT APPLN. NO. 09/890,646 **PATENT**
RESPONSE UNDER 37 C.F.R. §1.111 **NON-FINAL**

In the event any additional fees are required, please also charge our Deposit Account No. 111833.

Respectfully submitted,

KUBOVCIK & KUBOVCIK



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Checks for $465.00 and $639.00

[NAME OF DOCUMENT] PATENT APPLICATION

[CASE NUMBER] PA99001

[IPC] C12N 15/00

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SPECIFICATION 1

DRAWING 1

ABSTRACT 1

COPY OF RECEIPT OF MICROORGANISM 1

[NAME OF THE DOCUMENT] SPECIFICATION

[TITLE OF THE INVENTION] POLYNUCLEOTIDE ENCODING 2-HYDROXYISOFLAVANONE SYNTHASE

[CLAIMS]

1. 2-hydroxyisoflavanone synthase having an amino acid sequence which substantially comprises the amino acid sequence shown as SEQ-ID-No.:2.

2. A polynucleotide substantially comprising a nucleotide sequence encoding the 2-hydroxyisoflavanone synthase of Claim 1 or a nucleotide sequence complementary thereto.

3. A polynucleotide substantially comprising a nucleotide sequence which has 50% or more homology to a nucleotide sequence comprised in SEQ-ID-No.:1, and encodes 2-hydroxyisoflavanone synthase or the nucleotide sequence complementary thereto.

4. A polynucleotide which can be hybridized to at least a part of a nucleotide sequence in a coding region of SEQ-ID-No.:1 under stringent conditions .

5. A polynucleotide which can be hybridized to at least a part of a nucleotide sequence in a coding region of SEQ-ID-No.:1 under mild conditions.

6. A polynucleotide which substantially contains a nucleotide sequence having 50% or more of homology to the nucleotide sequence included in SEQ-ID-No.:1, and which can function as a probe or a primer for a polynucleotide substantially comprising a nucleotide sequence encoding 2-hydroxyisoflavanone synthase or a complementary nucleotide sequence to the nucleotide sequences.

7. A polynucleotide which substantially contains a nucleotide sequence having 70% or more homology to a nucleotide sequence consisting of at least 15 contiguous nucleotides included in SEQ-ID-No.:1, and which may function as a primer or a probe for a polynucleotide encoding 2-hydroxyisoflavanone synthase and a polynucleotide having a complementary sequence to the

nucleotide sequences.

8. A recombinant DNA or RNA containing an expression system which may express in a host cell a polynucleotide substantially comprising the nucleotide sequence encoding 2-hydroxyisoflavanone synthase according to Claim 1 or a nucleotide sequence complementary thereto.

9. A host cell containing one of the recombinant DNA or RNA according to Claim 8.

10. A method for producing 2-hydroxyisoflavanone synthase according to Claim 1 comprising culturing the host cell according to Claim 9.

11. The method according to Claim 10 further comprising a step of collecting the produced 2-hydroxyisoflavanone synthase.

12. A transgenic plants obtained by transforming the plant so that 2-hydroxyisoflavanone synthase, a product of an enzyme reaction catalyzed by 2-hydroxyisoflavanone synthase or derivatives thereof may be produced by introducing the recombinant DNA or RNA according to Claim 8 into a plant cell.

13. A transgenic plants obtained by transforming the plant so that an amount of 2-hydroxyisoflavanone synthase, a product of an enzyme reaction catalyzed by 2-hydroxyisoflavanone synthase or derivatives thereof may be altered by introducing the recombinant DNA or RNA according to Claim 8 into a plant cell.

[DETAILED DESCRIPTION]

[0001]

[TECHNICAL FIELD]

The present invention relates to a polynucleotide containing a newly identified nucleotide sequence encoding 2-hydroxyisoflavanone synthase, and the transformant transformed with the polynucleotide.

[0002]

[DESCRIPTION OF THE PRIOR ART]

Isoflavones are compounds having an isoflavone skeleton which are contained predominantly in a leguminous plants. It is known that isoflavones will act as a phytoalexin in plants. A phytoalexin is an antibacterial substance which plants produce against stresses such as microorganism infection.

Moreover, isoflavones, especially daidzein and genistein are physiologically active substances having an estrogen-like activity, which are notable for an effect of preventing a breast cancer or osteoporosis.

Moreover, there are also substances which attract an attention as powerful antioxidation substances, such as 6, 7, 4'- trihydroxy isoflavone, 7, 8, 4'-trihydroxy isoflavone and the like.

On the other hand, there was a case that a problem of sterilization of livestock arose, when leguminous plants which is rich in isoflavones has been used as food for livestock.

Therefore, there have been noted the possibility of control of an amount of the isoflavones produced in plants for the purpose of improvement in disease resistance of plants and production of isoflavones suitable as feed of livestock or the like.

[0003]

An isoflavone skeleton is biosynthesized via the oxidative aryl migration from flavanones to 2-hydroxyisoflavanones, as shown in Fig.1, it is known that the enzyme catalyzing the reaction, i.e., 2-hydroxyisoflavanone synthase exists. Therefore, 2-hydroxyisoflavanone synthase is a very important enzyme in synthesis of isoflavones.

[0004]

[PROBLEM(S) TO BE SOLVED BY THE INVENTION]

Although isolation and purification of 2-hydroxyisoflavanone synthase, and elucidation of an amino acid sequence of 2-hydroxyisoflavanone synthase and the DNA sequence encoding the amino acid sequence are important for production of isoflavones having the above-mentioned useful activity and control of the amount thereof produced in plants, isolation and purification of 2-hydroxyisoflavanone synthase and elucidation of cDNA sequence have not been realized.

[0005]

[MEANS FOR SOLVING THE PROBLEM]

In order to isolate cDNA of 2-hydroxyisoflavanone synthase and to determine the DNA sequence and the amino acid sequence thereof, the inventors have studied plant materials, a culture

condition, a mRNA induction, and the like, and as a result, have succeeded in cloning the cDNA encoding 2-hydroxyisoflavanone synthase from the cDNA library produced from the cells obtained at 6 to 12 hours after elicitor treatment of the callus culture of a licorice (Glycyrrhiza echinata) with yeast extracts.

[0006]

Accordingly, the present invention relates to 2-hydroxyisoflavanone synthase which has an amino acid sequence substantially comprising the amino acid sequence shown as SEQ-ID-No.:2.

[0007]

The present invention relates to a polynucleotide substantially comprising a nucleotide sequence encoding the above-mentioned 2-hydroxyisoflavanone synthase or a nucleotide sequence complementary thereto.

[0008]

In an another aspect, the present invention relates to a polynucleotide substantially comprising a nucleotide sequence which has 50% or more, preferably 70% or more, more preferably 80% or more, further preferably 90% or more and especially 95% or more of homology to the nucleotide sequence comprised in SEQ-ID-No.:1, and encodes 2-hydroxyisoflavanone synthase or the nucleotide sequence complementary thereto.

[0009]

Furthermore, in an another aspect, the present invention relates to a polynucleotide which can be hybridized to a nucleotide sequence in a coding region of SEQ-ID-No.:1 under stringent conditions or under mild conditions.

The mild hybridization condition and the stringent hybridization condition are described, for example, in Sambrook et. al, Molecular Cloning, A Laboratory Manual, 2nd. Vol.1, pp.1.101-104, and Cold Spring Harbor Laboratory Press, (1989).

[0010]

Furthermore, the present invention also relates to a polynucleotide encoding 2-hydroxyisoflavanone synthase obtained by cloning using a polynucleotide set forth in Claim 6 or 7 as a probe from the cDNA library produced from the cells 3 - 6 hours, preferably 1 to 12 hours, especially 1 to 8 hours after elicitor treatment of the cells of licorice, preferably Glycyrrhiza echinata. The elicitor treatment is preferably performed using the yeast extract.

[0011]

Moreover, the present invention relates to a polynucleotide having homology of 50% or more, preferably 70%, still preferably 80%, further preferably 90 % or more, and especially 95% or more to a nucleotide sequence contained in SEQ-ID-No.:1 which can function as a probe or a primer for a polynucleotide encoding 2-hydroxyisoflavanone synthase, especially a polynucleotide which can function as such a probe or a primer having homology of preferably 70% or more, more preferably 80% or more, still more preferably 90 % or more, especially 95% or more to the nucleotide sequence consisting of at least 15 contiguous nucleotides in SEQ-ID-No.:1.

[0012]

Moreover, the present invention also relates to a recombinant DNA or RNA containing the expression system which may express in a host cell a polynucleotide substantially comprising a nucleotide sequence encoding 2-hydroxyisoflavanone synthase or a nucleotide sequence complementary to the nucleotide sequence, a host cell containing the recombinant DNA or RNA, and a method for producing 2-hydroxyisoflavanone synthase comprising culturing the host cell. The method for producing may further include a step of collecting the produced polypeptides.

Furthermore, the present invention relates to a transgenic plants obtained by transforming the plant so that 2-hydroxyisoflavanone synthase, a product of an enzyme reaction catalyzed by 2-hydroxyisoflavanone synthase or derivatives thereof may be produced, or the amount thereof may be altered.

Moreover, the monoclonal antibody and a polyclonal antibody against the polypeptide of the present invention can be manufactured by the method well known in the art, and the present invention also relates to the antibodies.

[0013]

[EMBODIMENT OF THE INVENTION]

1. Definition

For easier understanding of the specification, a terminological definition will be shown below.

In the specification, "2-hydroxyisoflavanone synthase (hereafter referred to as IFS)" means the polypeptide having the enzyme activity of producing 2-hydroxyisoflavanon from flavanones as a substrate by a hydroxylation reaction and an aryl migration, when the procedure usually used for analysis of function of cytochrome P450.

Namely, it is meant that, for example, when the polypeptide is expressed in eucaryocytes,

such as yeast, the microsom has the activity of catalyzing such a reaction under existence of a NADPH coenzyme or the like and under aerobic conditions, or is the polypeptide having the activity of catalyzing such a reaction under the above-mentioned condition when it is mixed with phosphatide such as P450 reductase and dilauryl phosphatidylcholine to reconstruct an electron transport system.

[0014]

IFS according to the present invention has an amino acid sequence substantially comprising the amino acid sequence shown as SEQ-ID-No.:2.

"Substantially comprising the amino acid sequence shown as SEQ-ID-No.:2" means that the amino acid sequence may include variation(s) such as deletion, substitution, addition and insertion in the amino acid sequence. That is, IFS of the present invention may contain such variation, as long as the above-mentioned enzyme activity is maintained. The number of amino acids which are deleted, substituted, added or inserted may be, for example 1-20, preferably 1-10 and especially 1-5. For example, an amino acid residue can be replaced by a different amino acid residue with the similar characteristics. The typical substitution may be the substitution between Ala, Val, Leu, and Ile, between Ser and Thr, between the acid residue Asp and Glu, between Asn and Gln, between the basic residue Lys and Arg, or between the aromatic residue Phe and Tyr.

Furthermore, the present invention also relates to a polypeptide having an antigen activity of IFS set forth in Claim 1.

IFS of the present invention may be either isolated polypeptides which exists naturally, those produced by gene recombination technology, or those synthesized by known technology in the art.

Moreover, IFS of the present invention includes the polypeptide originated from Glycyrrhiza echinata , the polypeptide originated from other species belonging to Glycyrrhiza genus, the polypeptide originated form other genus belonging to a leguminous family, or the polypeptide originated from plants belonging to other families.

[0015]

In this specification, "the polynucleotide of the present invention" may be polynucleotides set forth in any one of Claims 2 to 7 as described above.

[0016]

The transformed Escherichia coli strain K12 CYP Ge-8 carrying the plasmid containing the polynucleotide encoding IFS of the present invention has been deposited with the Kogyo Gijutsuin Seimei-Kogaku Kogyo Gijutsu Kenkyujo of the Japan 1-1-3, Higashi, Tsukuba-shi, Ibaraki-ken on

February 1, 1999, and was accorded th accession number FERM P-17189. The present invention also relates to a polynucleotide comprising nucleotide sequence encoding IFS obtained by a conventional method from of Escherichia coli strain K 12 of the accession number FERM P-17189.

[0017]

The "polynucleotide" includes poly ribonucleotides and poly deoxyribonucleotides, and may be unmodified RNA and DNA or modified RNA and DNA.

In the present invention, the "polynucleotide" may be those isolated from the naturally existing state. Moreover, in the present specification, the word "polynucleotide" include single strand DNA, double strand DNA, DNA comprising single strand part(s) and double strand part(s), single strand RNA, double strand RNA, RNA comprising single strand part(s) and double strand part(s), hybrid molecules containing DNA and RNA each of which may be single strand or double strand, or may comprise single strand part(s) and double strand part(s).

The word "polynucleotide" also include DNA or RNA containing one or more modified nucleotides, and DNA or RNA having the skeleton modified for stability or other reasons.

The word "modified base" include a tritylated base or a base like an inosine. Therefore, a "polynucleotide" may be a polynucleotide modified chemically, enzymatically, or metabolically.

In the present specification, the word "polynucleotide" may also include an oligonucleotide.

[0018]

The word "polypeptide" means the peptide or protein containing two or more amino acids bonding each other through a peptide bond or a modified peptide bond. The word "polypeptide" means both a short chain (so-called peptide, an oligopeptide, or oligomer) and a long chain (protein).

The "polypeptide" includes the amino acid sequence modified by the natural process like posttranslational-modification processing, or by the chemical-modification method well known in the art. Such a modification is well known in the art. The modification can may take place in any place of the polypeptide including a peptide skeleton, an amino acid side chain, an amino terminus, and a carboxyl terminus. A polypeptide may include many types of modification. The polypeptide may be branched as a result of ubiquitination, or may be cyclized with or without being branched. Modification may include: an acetylation, an acylation, ADP ribosylation, an amidation, covalent bonding with a flavin, the covalent bonding with a heme part, nucleotide, or the covalent bonding with a nucleotide inductor, the covalent bonding with a lipid or a lipid inductor, the covalent bonding with a phosphotidil inositol, formation of the cross linkage, cyclization, formation of a disulfide bond,

demethylation, and formation of a cross linkage by covalent bond, formation of a cystine, formation of a pyroglutamic acid, formylizing, a gamma carboxylation, glycosylation, GPI anchor formation, hydroxylation, iodine-izing, a methylation, myristoylation, oxidization, protein decomposition-processing, phospholylation, prenylation, racemization, selenoylation, sulfation, and amino acid addition to protein by transfer RNA such as argininization, and ubiquitinationation.

[0019]

The word "antisense inhibition" means inhibiting expression of a target gene by antisense RNA which is complementary to all or a part of the target primary transcript product or mRNA, and inhibiting processing, movement, and/or translation of the target primary transcript product or mRNA. The antisense RNA may be complementary to any part, i.e., 5 'non coding sequence, 3' non coding sequence, intron, or coding sequence of a specific gene transcript product. In addition, the antisense RNA as used herein can include the domain of the ribozyme sequence which increases the effect of inhibiting gene expression by an antisense RNA.

"Ribozyme" means a catalytic RNA, including endoribonuclease which is specific to a sequence. "The suitable regulatory sequence" used in the present specification means natural or chimeric nucleotide sequence in a gene which is located in the upstream (5'), an inside and/or downstream (3') of the nucleic acid fragment of the present invention, and controls expression of the nucleic acid fragment of the present invention.

"3' non coding sequence" means the DNA sequence part of a gene including polyadenylation signal, and any other regulation signal which can affect processing of mRNA or the gene expression. The polyadenylation signal is usually characterized by affecting addition of the polyadenylic-acid part to the 3'-end of a mRNA precursor.

"Plants" means photosynthetic organisms of both an eukaryotic organism and a prokaryotic organism.

Although the polynucleotide of the present invention was explained in various expression in the present specification, it is intended that the polynucleotide of the present invention includes all the nucleotide sequences which can be obtained by the skilled in the art using the procedure known in the art based on the information on cDNA sequence which has been elucidated by the present invention, and of which IFS activity can be confirmed, and also includes all the nucleotide sequences that are used as a probe or a primer used based on the information in order to obtain IFS.

[0020]

2. Production of Transformant, and production of IFS

The transformant of the present invention is obtained by introducing the recombinant DNA or RNA of the present invention into the host suitable for the expression vector used when producing these recombinant DNA or RNA. The purified polynucleotide is inserted in the restriction enzyme part or multi-cloning site of a suitable vector to provide the recombinants DNA or RNA, and the host cell is transformed using the recombinants DNA or RNA concerned.

The vector DNA in which a DNA fragment is inserted will not be limited as far as it can be replicated in a host cell, for example, plasmid DNA, phage DNA, or the like. Examples of the plasmid DNA may include: plasmids pUC118 (manufactured by TAKARA SHUZO CO., LTD.) and pUC119 (manufactured by TAKARA SHUZO CO., LTD.), pBluescript SK (+) (manufactured by Stratagene), pGEM-T (manufactred by Promega), and the like. Examples of the phage DNA may include M13mp18, M13mp19 and the like.

The host will not be limited, as far as it can express the target gene, and both an eukaryotic cell and a prokaryotic cell can be used as the host, but an eukaryotic cell is used preferably. For example, there can be used bacteria such as Escherichia coli and Bacillus subtilis, yeast such as Saccharomyces cerevisiae, an insect cell, animal cells such as a COS cell, and a CHO cell and the like.

[0021]

When using bacteria such as Escherichia coli as a host, the recombinants DNA or RNA of the present invention is preferably autonomously replicable in the host, and preferably has a constitution comprising a promoter, polynucleotide of the present invention and a transcription termination sequence. For example, Escherichia coli can be XL1-Blue (manufactured by Stratagene), JM109 (manufactured by TAKARA SHUZO CO., LTD.), or the like, and an expression vector can be, for example, pBTrp2 or the like. As the promoter, any promoter can be used as long as it can be expressed in hosts, such as Escherichia coli. For example, there can be used promoters originated from Escherichia coli, phages, or the like, such as a trp promoter, a lac promoter, PL promoter, and PR promoter. A transformation can be performed, for example, by the procedure of Hanahan [Techniques for Transformation of E.coli In DNA Cloning, vol.1, Glover, D.M.(ed.) pp 109-136, and IRLPress (1985)].

[0022]

When using yeast as a host, YEp13, YCp50 or the like can be used as an expression vector. As a promoter, gal 1 promoter, gal 10 promoter, or the like can be used, for exampl . As a method

for introducing the recombinants DNA or RNA into yeast, there can be used, for example, the electroporation method (Methods.Enzymol., 194,182-187 (1990)), the spheroplast method (Proc.Natl.Acad.Sci.USA, 84, 1929-1933 (1978)), the acetic acid lithium method (J. Bacteriol., 153,163-168 (1983)) or the like.

[0023]

When using an animal cell as a host, pcDNAI, pcDNAI/Amp (invitrogen) or the like can be used as an expression vector. As method for introducing the recombinant DNA or RNA to an animal cell, an electroporation method, a calcium phosphate method, or the like can be used, for example.

In the case that plasmid DNA is used as a vector, for example, in order to insert EcoRI DNA fragment into plasmid DNA, the plasmid DNA is previously digested using the restriction enzyme EcoRI (manufactured by NEB). Subsequently, a DNA fragment and the digested vector DNA are mixed, which is then affected by T4DNA ligase (manufactured by TAKARA SHUZO CO., LTD.) to provide a recombinant DNA.

[0024]

Screening of the above-mentioned transformant can conducted by selecting the colony containing the targeted gene according to colony hybridization using the DNA fragment containing a part of the targeted gene as a probe or by PCR method using as a primer 5'-primer synthesized based on the nucleotide sequence of the target gene, and 3'-primer synthesized based on the nucleotide sequence of a complementary strand, and can choose the colony containing the target gene by the PCR method using these primers.

[0025]

If the transformant carrying the recombinant DNA or RNA obtained as mentioned above is cultured, the polypeptide of the present invention can be produced. The usual solid-culture method is sufficient as a culturing method, but it is desirable to adopt a liquid culture method.

As a medium in which a transformant is cultured, there can be used a medium wherein one or more kinds of mineral salts such as potassium phosphate, magnesium sulfate and ferric chloride are added to one or more sorts of nitrogen sources chosen, for example from a yeast extract, peptone, a meat extract, or the like, and if desired, a saccharine material, antibiotics, the vitamin, or the like is added thereto. Moreover, IPTG or the like may be added to the medium to induce expression of a gene, if desired. pH of the medium is adjusted to 7.2-7.4 when culturing is initiated, and a general aeration spinner culture method, a shaking culture method or the like may be conducted at preferably

36-38 °C, preferably around 37 °C for 14 to 20 hours.

[0026]

After a culturing is terminated, IFS of the present invention can be extracted from the culture according to a general method for purifying protein. That is, after destroying a cell and carrying out the solubilization of the IFS in the presence of a surfactant by the lysis treatment using enzymes, such as lysozyme, ultrasonic spallation treatment, grinding treatment, or the like, IFS are discharged from the cells. Subsequently, an insoluble substance is removed using filtration or a centrifugal separation to provide a rough polypeptide solution.

IFS can be further purified from the above-mentioned rough polypeptide solution by the general method for purifying protein using a surfactant suitably. For example, an ammonium sulfate salting-out method, an ion exchange chromatography, hydrophobic chromatography, gel-filtration chromatography, an affinity chromatography, an electrophoresis, or the like can be conducted solely or in combination.

IFS obtained by the above-mentioned procedure can be used to synthesize 2-hydroxy isoflavanone according to hydroxylation reaction and an aryl migration from flavanones as a substrate after forming a micelle in the presence of P450 reductase and phosphatide such as dilauryl phosphatidylcholine. When a transformant is an eucaryocyte, the microsome obtained by crushing the cell obtained by culturing after a transformation and subjecting it to a centrifugal separation can be used to synthesize 2-hydroxy isoflavanone by the same reaction. The present invention also relates to use of the polynucleotide of the present invention for manufacture of the compound obtained by the reaction of the enzyme of the present invention or derivatives thereof. Examples of such compounds or derivatives thereof include: daidzein, genistein, 6, 7, 4'-trihydroxy isoflavone, 7, 8, 4'-trihydroxy isoflavone, formononetin, and 2'-hydroxy formononetin, medicarpin or the like.

[0027]

3. Transgenic Plants

There can be obtained transgenic plants toransformed so that IFS, the product of the enzyme reaction catalyzed by IFS or its derivatives may be produced by introducing the polynucleotide encoding IFS of the present invention with the transcription regulatory region which can express in the plant cell such as a suitable promoter, a terminator, or the like into a plant cell. Thereby, it is made possible to express IFS in plants which does not express IFS originally, and thereby to made the plants can be made to produce 2-hydroxy isoflavanones, which is the product of the reaction catalyzed

thereby and the derivatives thereof. Examples of the isoflavones can be those listed above. Therefore, the present invention relates also to use of the polynucleotide for obtaining plants which produces these substances. Such a method is useful for raising disease resistance of the plants by making the plant that does not contain isoflavones originally to express isoflavones, or for providing plants as foods which contains a large amount of isoflavones.

[0028]

Examples of a transcription regulatory region which can be expressed in the plant cell include CaMV35SRNA promoter, a CaMV19SRNA promoter, a nopaline-synthesis enzyme promoter or the like expressed in the whole plants mentioned above, , RubisCO small-subunit promoter expressed in green organizations, the promoter region of genes expressed in specific part such as seeds, for example, napin and phaseolin, or the like. Moreover, terminators such as a nopaline-synthesis enzyme terminator and a RubisCO small-subunit 3'-end part may be connected with 3'-end.

Moreover, expression can be increased by introducing an enhancer into a promoter region. Examples of an enhancer include: the enhancer of a virus as found in 35S promoter(Odell et al., Plant Mol.Biol.(1988)10:263-272), the enhancer from an opine gene (Fromm et al., Plant Cell 1(1989):977-984), and any other enhancers which may increase transcription when located in the promoter connected so that it can act the polynucleotide of the present invention. Thereby, the transgenic plants wherein the content of isoflavones is high can be obtained. Examples of isoflavones were listed above. Therefore, the present invention relates also to use of the polynucleotide of the present invention for providing plants wherein a production amount of isoflavones is increased. Such a procedure is useful to provide plants of which disease resistance was raised, plants provided as food containing isoflavones in a large amount.

[0029]

Furthermore, according to the present invention, the expression of IFS in plants can be controlled by anti sense inhibition. That is, expression of IFS can be inhibited by introducing into a plant cell the vector containing the antisense RNA of the polypeptide of the present invention with an expression-control sequence such as a promoter. Thereby, the transgenic plants containing no or a reduced amount of isoflavones can be obtained. Examples of isoflavones were listed above.

Therefore, the present invention also relates to use of the polynucleotide for obtaining plants wherein production of these substances was controlled. This procedure is useful, for example, to provide feed which does not cause sterilization of livestock.

[0030]

Transgenic plants can be obtained by the procedure widely known in the art, for example, the Agrobacterium method, the particle gun method, the electroporation method, the PEG method, or the like., and the method of being suitable for a host cell is chosen.

An example of the Agrobacterium method is a method using a binary vector. The method comprises transfecting a plant with a vector containing T-DNA originated from the Ti plasmid origin, a replication origin which can act in a microorganism such as Escherichia coli and a marker gene for choosing the plant cell or the microbial cell carrying the vector, growing the seed harvested from the resultant plants, and selecting transformed plants wherein the vector was introduced using expression of the marker gene as an index. The transformed plants to be intended can be obtained by measuring IFS activity or selecting the plants wherein contents of isoflavones as a product of the reaction catalyzed by IFS or a derivative thereof is altered (Plant Physiol., 91 and 1212 (1989), WO 94/02620, Plant Mol.Biol., 9, 135 (1987), Bio/Technology, 6, 915 (1988)).

Moreover, the particle gun method is performed by a procedure described in the following reference: Pro.Natl.Acad.Sci. USA, 86, 145 (1989), TIBTECH, 8, 145 (1990), Bio/Technology, 6, 923 (1988), Plant Physiol., 87, 671 (1988), Develop. Genetics, 11, 289 (1990), Plant cell Tissue & Organ Culture, 33, 227 (1993).

The electroporation method can be performed by the procedure described in the following reference: Plant Physiol., 99, 81 (1992), Plant Physiol., 84, 856 (1989), Plant Cell Reports, 10, 97 (1991).

[0031]

[Example]

The present invention will be further explained by the following Examples. Unless being indicated otherwise, all ratios and percentages are based on weight. These Examples are for illustration, and do not limit the present invention. The skilled in the art can make various improvement and modification using the description of the present invention and information known in the art.

[0032]

1. Plant Materials and Culture Methods

The callus cultures were stablished from leaves and petiole of Glycyrrhiza echinata (hereafter referred to as a licorice). The callus was grown on half-strength Murashige-Skoog's

medium (solidified with 0.3% (w/v) of gellan gum) containing α-naphthalene acetic acid (1 mg/l) and an N6-benzyladenine (1 mg/l), under 12 hours light (6,000 luxs) and 12 hours dark cycle. The cultures was suspended and cultured in MS medium to which 2,4-dichlorophenoxy acetic acid (0.1 mg/l) and the kinetin (1 mg/l) were added. The culture was subjected to elicitor treatment by adding 0.2% (w/v) of yeast extract (YE, manufactured by Difco). Then, the cultured cells were collected by vacuum filtration, frozen in liquid nitrogen immediately, and saved at -80 ° C.

[0033]

2. cDNA library and Screening

RNA having poly (A) was isolated from the cultured cell of the above-mentioned licorice 6 hours and 12 hours after the elicitor treatment, using Straight A's mRNA isolation system (manufactured by Novagen). The RNAs having poly (A) (2.5 μg each) were mixed, cDNA corresponding thereto were prepared using the ZAP-cDNA Synthesis Kit (manufactured by Stratagene) to construct cDNA library. Plaques (2×10^5) of the cDNA library were transferred on High-bond N+ membrane (manufactured by Amersham), and screened using as a probe polynucleotide having a sequence of SEQ ID No.3 labeled with horseradish peroxidase (hereinafter referred to as HRP) using an ECL direct nucleic acid labeling system (manufactured by Amersham). The polynucleotide is one of segments obtained from the cultured cell of the licorice 8 hours after the elicitor treatment as the above by PCR (95 °C for 3 minutes, then 30 cycles of 95 °C for 1 minute, 45 °C for 1 minute and 72 °C for 2 minutes, and finally 72 ° C for ten minutes) using as the primers the synthetic polynucleotides of: 5'-(T/C/A) TI(C/G) CITT(T/C) (G/A) GIIIGGI(A/C) (G/C) I(A/C) G-3' (I is inosine) (SEQ ID No. : 8) and 5'-AATACGACTCACTATAG-3' (SEQ ID No. : 9). Hybridization was performed using ECL hybridization buffer containing 500 mM NaCl and 5 % blocking reagent at 42 ° C for six minutes. The membranes were washed twice with 1 x SSC containing 0.4 % SDS at 55° C for 10 min and twice with 2 x SSC at room temperature for 10 minutes. ECL detection reagent (manufactured by Amersham) was added to the membrane to detect HRP-labelled hybrid composite. The membrane was exposed to Kodak XAR-5 film for one minute. Possible clones were inserted to pBluescript SK(-) phagemids by in vivo excision according to the manufacturer's protocols. The length of the inserted cDNA was determined by PCR using T3 (5'-ATTAACCCTCACTAAAG-3' (SEQ ID No. : 10)) and T7 (5'-AATACGACTCACTATAG-3' (SEQ ID No. : 9)) primers, and the complete nucleotide sequence of the clones which had a length of about 2000 bp were determined. One of them was cDNA having a s quence of SEQ ID No.:1, and

identified to be IFS according to the following procedures (hereinafter the complete nucleotide sequence cDNA is referred to as CYP Ge-8).

[0034]

3. Construction of expression vectors, expression in yeast cells and preparation in microsomes

The coding region of CYP Ge-8 was amplified by PCR using KOD polymerase, CYP Ge-8 cDNA clone as the template and the following sense primer (Ge-8S1, SEQ ID NO.:4) and antisense primer (Ge-8A1, SEQ ID No.:5). The sequences of the specific primer were as follows:

Ge-8S1: 5'-AAACAGGTACCATGTTGGTGGAACTTGC-3'

Ge-8A1: 5'-CGCGCGAATTCTTTACGACGAAAAGAGTT-3'

The sense primer has KpnI site (GGTACC) upstream of the initiation codon (ATG) and EcoRI site (GAATTC) downstream of the termination codon (TAA). The KpnI-EcoRI fragment of the PCR product was cloned into corresponding sites of pYES2 expression vector (manufactured by Invitrogen) having URA3 selection marker. The protease deficit yeast (Saccharomyces cerevisiae) BJ2168 strain (a;prc 1-407, prb 1-1122, pep 4-3, leu2, trp1, and ura3-52 manufactured by Nippon gene corporation) was transformed with the plasmid pYES Ge-8 thus obtained according to the acetic acid lithium method. The transformant was selected in culture medium containing 6.7 mg/ml of yeast nitrogen base without amino acids(manufactured by Difco), 20 mg/ml of glucose, 30 μg/ml of leucine, 20 μg/ml of tryptophan, 5 mg/ml of casamino acid.

[0035]

The transformed yeast was cultured in the above-mentioned liquid medium (25ml) for 10 hours for the induction of P450 protein, and cells were collected by the centrifugal separation (3,000 x g). The cells were transferred to 1000ml (40 times) of the YPGE medium which does not contain glucose (10 g/l yeast extract (manufactured by Difco), 10 g/l of bactopeptone (manufactured by Difco), 20 g/l of galactose, 3% (w/v) of ethanol, and 2 mg/l of hemin), and cultured for 24 hours and 36 hours. The collected yeast cells were suspended in 0.1 M potassium phosphate (pH 7.5) containing 10% sucrose and 14 mM of 2-mercaptoethanol with glass beads (diameter of 0.35 to 0.6 mm), and disrupted by a vigorous shaking using a vortex mixer for 10 min. They were subjected to centrifugation procedures of 10,000 x g and 15,000 x g for 10 minutes, and the obtained supernatant was applied to ultracentrifugation procedures of 160,000 x g for 90 minutes to prepare the microsome. The yeast cell transformed with pYES2 was used as control.

[0036]

4. Enzyme assay

a) Manufacture of a substrate

As radio labelled substrate for enzyme assay, (2S)-[^{14}C]liquiritigenin (7,4'-dihydroxyflavanone) and (2S)-[^{14}C]naringenin (5,7,4'-trihydroxyflavanone) were prepared. (2S)-[^{14}C]liquiritigenin (7,4'-dihydroxyflavanone) was prepared by incubating malonyl-CoA and the 4-cumaroyl CoA with the cell free extract of the licorice cultured cell 12 hours after elicitor treatment and NADPH at 30 °C for 3 hours.

Moreover, (2S)- [^{14}C]naringenin (5,7,4'-trihydroxyflavanone) was prepared by the same method as the above except that NADPH was not added.

The resulting (2S)-[^{14}C]liquiritigenin (hereinafter referred to as the labelled liquiritigenin) or (2S)-[^{14}C]naringenin (hereinafter referred to as the labelled naringenin) were purified with TLC (6.4 kBq/nmol each, 0.08 nmol).

[0037]

b) Reaction with the labelled liquiritigenin

The labelled liquiritigenin purified above was dissolved in 30 µl of 2-methoxyethanol, added to 1 ml of the above yeast microsome, and was incubated with in the presence of 1 mM NADPH at 30°C for 2 hr.

After the reaction was terminated with 30 µl acetic acid, the ethyl acetate extract in the mixture was analyzed by TLC-radiochromatoscanner.

TLC was developed with 15% acetic acid using the cellulose (manufactured by Funakoshi corporation, Funacel SF). The result is shown in Fig. 2A. From the chart, the existence of three radioactive compounds [P1 (Rf0.74), P2 (Rf0.64), P3 (Rf0.38)] other than an unreacted substrate (Rf 0.51) was confirmed. From Rf value, P3 was determined with high possibility to be an isoflavone, namely daidzein (7, 4'-dihydroxy isoflavone.

For acid-catalyzed conversion of the reaction products into isoflavones, the concentrated ethyl acetate extracts were dissolved in 500 µl of 10% HCl in methanol, and stirred at room temperature for 1 h and 50°C for 10 min. The reaction mixture was extracted with ethyl acetate, and the product was subjected to TLC under the same condition, and then analyzed by TLC radiochromatoscanner. The result is shown in Fig. 2B. As shown in the chart, the relative radioactivity at the Rf of P1 decreased and that of P3 increased. Accordingly, P1 is highly possible to be 2,7,4'-trihydroxyisoflavanone, which should readily be dehydrated to give daidzein.

[0038]

Furthermore, the radioactive P1 was isolated from the TLC plate of which autoradiogram was shown in Fig.3A, reacted with HCl, and analyzed by TLC autoradiography. Thereby, a pure radioactive product P3 (daidzein) was produced as shown in Fig.3B.

To further confirm that CYP Ge-8 is IFS, 10 μg of liquiritigenin without radiolabel was added to one ml of the above microsome and incubated at 30 °C for 2 hours in the presence of 1 mM of NADPH. The reaction mixture was extracted with ethyl acetate, and the ethyl acetate phase was analyzed by HPLC using a Shim-pack CLC-ODS column (6.0 x 150 mm; manufactured by Shimadzu) with 40% methanol in H_2O. The eluent was monitored at 285 nm. The result was shown in Fig.4A. As expected, a peak of daidzein (Rt 21.0 min) and two additional peaks (Rt 5.5 and 7.9 min) were observed. When the ethyl acetate extract was treated with acid, an intense daidzein peak was observed as shown in Fig.4B. The product giving Rt 5.5 min peak in Fig. 4A was proven to be P1 by TLC analysis.

[0039]

For mass spectrometric analysis, the incubation of the above yeast microsome with liquiritigenin was carried out in a large scale (x 200 of the scale described above). The ethyl acetate extract of the reaction mixture was applied to TLC for separation on Kieselgel F254 (manufactured by Merck) using as solvent toluene/ ethyl acetate/ methanol/ petroleum benzine (6: 4: 1: 3). The spots at the P1 (Rf 0.2) and P3 (Rf 0.3) spots were collected and further purified by HPLC. The mass spectra were recorded on a JEOL JMS-AX505H mass spectrometer under the electron impact (EI) mode with the ionization voltage of 70 eV.

[0040]

c) Reaction with labeled naringenin

An incubation with a yeast microsome and TLC analysis were performed by the same procedure as the above except that the labeled naringenin was used instead of the labeled liquiritigenin as a substrate. As shown in Fig. 7A, P4 (Rf0.69) and P5 (Rf0.64) were confirmed as radioactive spots. Then, the spots of P4 were collected, and treated with HCl. The new spots were obtained as shown in Fig. 7B. These spots showed the same mobility as the sample of genistein. Therefore, it was confirmed that P4 was a 2, 5, 7, 4'-tetra hydroxy isoflavone.

The above-mentioned experiment proved that the protein encoded by CYP Ge-8 acts both liquiritigenin and naringenin, which may produce 2, 7, 4 '-trihydroxy isoflavanone and 2, 5, 7, 4'-tetrahydroxy isoflavanone respectively converting to daidzein and genistein by acid treatment, namely it was IFS Although dehydration was performed by

HCl treatment in the above Example, it is thought that dehydration progresses by the action of a dehydratase in the cell of a licorice.

[0041]

5. P450 antisera and immunoblot analysis

Consensus amino acid sequences of plant cytochrome P450 have been studied thoroughly, and two oligopeptide shown below were designed as an antigen: (Leu Pro Phe Gly Ser Gly Arg Arg Ser Cys (SEQ ID No. : 6) and Tyr Leu Gln Ala Ile Val Lys Glu Thr Leu Arg Leu (SEQ ID No. : 7)).

The multiple-antigen peptides with N-acetylated amino-terminals synthesized chemically based on these sequences were subcutaneously injected into two rabbits, and the anti-peptide antibody titers in blood were determined with ELISA, and it was revealed that the antibody against P450 was produced. Immunoblotting was conducted using the P450 antisera.

The above yeast microsome was separated on SDS/10% PAGE and transferred onto a Hybond-C membrane (manufactured by Amersham) (about 10 μg protein), and then incubated with a 1: 2000 dilution liquid of the P450 antiserum for 1 h at room temperature. The immunoreactive protein was detected by ECL Western blotting analysis system (manufactured by Amersham) according to a manufacturer's protocol.

[0042]

As shown in Fig. 8A, in SDS/PAGE, the new band having a molecular weight of about 59kDa was detected. It was in agreement with the value (59,428 Da) calculated based on the amino acid sequence.

As shown in Fig.8B, a prominent signal at 59 kDa was displayed with microsomes of the cells expected to express CYP Ge-8.

[0043]

6. Northern blot analysis

Suspension-cultured cells of licorice cultured under the same culturing condition as described in the paragraph "1. Plant material and culturing method" were harvested at 3, 6, 12, 24 and 48 h post-elicitation. mRNAs were extracted using Straight A's mRNA Isolation System (manufactured by Novagen). For northern blot analysis, mRNA (900 ng) was subjected to electrophoresis on 1% agarose-formaldehyde gel and transferred onto a Hybond-N+ membrane (manufactured by Amersham). An amount of RNA were determined by staining the gel after electroporation with

ethidium bromide. CYP Ge-8 coding region was amplified by PCR using the above-mentioned primers Ge-8S1 and Ge-8A1. Probes for the hybridization were prepared by labelling it by alkaline phosphatase using an AlkPhos Direct system for chemiluminescence (manufactured by Amersham). The blot was hybridized with the probes in the hybridization buffer containing 500 mM NaCl and 4% blocking reagent for 12 h at 55°C. The membranes were washed twice with the primary wash buffer at 55 °C for 10 min and twice with the secondary wash buffer for 5 min at room temperature according to the manufacturer's protocols.

As shown in a Fig. 9, it was revealed that there is much accumulation of mRNA in the cell at the time of 3 - 6-hour progress after elicitor treatment.

[0044]

[EFFECT OF THE INVENTION]

As explained above, the present invention provides the polynucleotide which contains substantially the nucleotide sequence encoding 2-hydroxyisoflavanone synthase. The transformant expressing IFS can be obtained using the polynucleotide. It is useful in production of isoflavones, supply of foods rich in isoflavones, improvement in plant disease resistance, or the like.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a reaction formula showing the reaction in which 2-hydroxyisoflavanone synthase of the present invention participates.

Fig. 2 is a chart showing the result of enzyme assay of the yeast microsome transformed with the polynucleotide of the present invention.

Fig. 3 is a photograph showing the result of enzyme assay of the yeast microsome transformed with the polynucleotide of the present invention.

Fig. 4 is a chart showing the result of enzyme assay of the yeast microsome transformed with the polynucleotide of the present invention.

Fig. 5 is a chart showing the result of mass-spectrum analysis of the reaction product by the yeast microsome transformed with the polynucleotide of the present invention.

Fig. 6 is a chart showing the result of mass-spectrum analysis of the reaction product by the yeast microsome transformed with the polynucleotide of the present invention.

Fig. 7 is a photograph showing the result of enzyme assay of the yeast microsome transformed with the polynucleotide of the present invention.

Fig. 8 is a photograph showing the result of SDS/PAGE and immunoblot analysis of a yeast microsome transformed with the polynucleotide of the present invention.

Fig. 9 is a photograph showing the result of Northern blot analysis of mRNA obtained by the present invention.

STATEMENT

I, Shizuko Kawamata, hereby state that I am competent in both the Japanese and English languages and that the attached English language document is an accurate translation of Japanese Patent Application No. 11-63745, filed February 4, 1999.

Date: July 4, 2003

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